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Most Influenza A Virions Fail To Express at Least One Essential Viral Protein

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Segmentation of the influenza A virus (IAV) genome enables rapid gene reassortment at the cost of complicating the task of assembling the full viral genome. By simultaneously probing for the expression of multiple viral proteins in MDCK cells infected at a low multiplicity with IAV, we observe that the majority of infected cells lack detectable expression of one or more essential viral proteins. Consistent with this observation, up to 90% of IAV-infected cells fail to release infectious progeny, indicating that many IAV virions scored as noninfectious by traditional infectivity assays are capable of single-round infection. This fraction was not significantly affected by target or producer cell type but varied widely between different IAV strains. These data indicate that IAV exists primarily as a swarm of complementation-dependent semi-infectious virions, and thus traditional, propagation-dependent assays of infectivity may drastically misrepresent the true infectious potential of a virus population.

Influenza A virus (IAV) poses a constant public health threat. Despite yearly production of seasonal influenza vaccines, IAV infections are responsible for an estimated average of 36,000 deaths and 200,000 hospitalizations annually in the United States alone, costing nearly $87 billion (1), and all of these figures rise dramatically with the introduction of pandemic strains every 20 years or so. Predicting the emergence of new epidemic or pandemic strains of IAV remains impossible, in large part because the viral determinants that govern pathogenicity and transmissibility in humans are poorly understood.

The IAV genome consists of eight negative-sense RNA segments (viral RNA [vRNA]), each essential for productive infection (2). Genome segmentation confers obvious evolutionary benefits by enabling high-frequency recombination during dual infection of host cells. Segment reassortment between zoonotic and human strains of IAV periodically generates novel viruses with pandemic potential that efficiently replicate in humans yet escape preexisting immunity (3–5). Segmentation, however, comes at the cost of requiring mechanisms to ensure cosegregation of all eight segments. It is widely believed that IAV is capable of producing a high fraction of virions with eight functional segments (6, 7, 31, 32).

Here, we provide contradictory evidence by demonstrating that the majority of individual IAV virions express an incomplete set of viral proteins and are thus incapable of multiround replication. Moreover, we observed considerable variation among different IAV strains, suggesting that the efficiency of incomplete infectious virion production may represent a novel determinant of transmission efficiency and pathogenicity.

MATERIALS AND METHODS

Cells and viruses. MDCK cells were maintained in minimal essential medium (MEM; Gibco) plus 7.4% fetal bovine serum (FBS), Vero cells were maintained in RPMI 1640 medium (Gibco) plus 7.4% FBS, and A549 cells were maintained in F-12 medium (Gibco) plus 7.4% FBS.

A/Puerto Rico/8/34 (PR8) (Mt. Sinai strain; H1N1), A/California/07/09 (Cal; H1N1), A/New Caledonia/20/99 (NewCal; H1N1), and A/Udorn/72 (Udorn; H3N2) were all propagated in 10-day-old embryonated chicken eggs. Where specified in the text, viruses were instead grown in MDCK cells. IAV titers were determined by standard 50% tissue culture infective dose ($TCID_{50}$) assay on MDCK cells. Multiplicities of infection (MOIs) were calculated based on $TCID_{50}$ titers. VSV Indiana strain was grown in BHK cells, and titers were determined by standard plaque assay on MDCK cells.

Animals. Animal studies were performed in accordance with an animal study proposal approved by the NIAID Institutional Animal Care and Use Committee. C57BL/6 mice were obtained from Taconic Laboratories. For mouse infections, 100 $TCID_{50}$s of virus in a volume of 25 μl of balanced sterile saline supplemented with 0.1% bovine serum albumin (BSS-BSA) was pipetted into the nostrils of mice anesthetized by isoflurane inhalation. At day 7, lungs were harvested and homogenized, and viral titers were determined.

For guinea pig infections, 12-week-old specific-pathogen-free female Hartley strain guinea pigs obtained from Charles River Laboratories were anesthetized by isoflurane inhalation and infected intranasally with 10^4 $TCID_{50}$s of PR8. Nasal washes were performed at 48 h postinfection (p.i.) to obtain virus at peak titer.

Immunofluorescence (IF). Cells grown on glass coverslips were infected with the IAV strains indicated in the figure legends and the indicated MOIs. At the times indicated, cells were washed with PBS and then fixed for 20 min with phosphate-buffered saline (PBS)–3.2% paraformaldehyde. After three washes with PBS, fixed cells were permeabilized with 1% Triton X-100 for 2 min at room temperature and then incubated in BSS-BSA for 5 min. Primary staining was carried out in BSS-BSA with 5% normal donkey serum (NDS) (Jackson ImmunoResearch). Anti-IAV mouse monoclonal antibodies (MAbs) used included the following: anti-H1, H36-26; anti-H3, H14-A2; antinucleoprotein (anti-NP), HB-65; antineuraminidase (anti-NA), NA2-1C1; anti-matrix protein (anti-M1), M2-1C6; and anti-nonstructural protein 1 (anti-NS1), 1A7 (all produced in-house). Other anti-IAV antibodies used included the following: rabbit anti-NA polyclonal antibody (pAb), rabbit anti-NA pAb, goat anti-M1/M2 pAb, and human anti-hemagglutinin (HA) stem MAb 70-1F02.

Received 27 August 2012 Accepted 24 December 2012
Published ahead of print 2 January 2013
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doi:10.1128/JVI.02284-12

After 1 to 2 h of primary staining at room temperature, cells were washed five times with PBS and then stained with secondary antibodies diluted in BSS-BSA plus 5% NDS. Secondary antibodies used included fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG, Texas Red-conjugated donkey anti-rabbit IgG, Dylight-549-conjugated donkey anti-rabbit, Cy5-conjugated donkey anti-goat IgG, and Cy5-conjugated donkey anti-human IgG (all Jackson ImmunoResearch). After 1 to 2 h of primary staining at room temperature, cells were washed three times with PBS and two times with water and then mounted on slides using Fluoromount-G (SouthernBiotech). After a drying step, slides were imaged on a Leica SP5 confocal microscope (Leica Microsystems).

Flow cytometry. To assess IAV protein expression by flow cytometry, cells were infected with PR8 at an MOI of 0.03, diluted in BSS-BSA. After 1 h of adsorption, inoculum was removed, cells were washed, and then MEM supplemented with FBS was added. One hour after the addition of medium, the neutralizing anti-HA MAb H17-L2 was added to a final concentration of 10 μg/ml. Infections were allowed to proceed for 17 h, at which point cells were harvested, washed, fixed, and permeabilized in FoxP3 fixation/permeabilization buffer (eBioscience). Culture supernatants were collected at the time of harvest and assessed to ensure the complete neutralization of free virus. After further washing in PBS–0.1% saponin, cells were blocked with Fc block (MAb 2.4G2; produced in-house) when necessary, and then stained with Alexa Fluor 488-conjugated anti-HA (mouse MAb H36-26), Pacific orange-conjugated anti-NA (MAb NA2-1C1), Alexa Fluor 647-conjugated anti-NP (mouse MAb HB-65; produced in-house), and Pacific blue-conjugated anti-NS1 (mouse MAb NS1-1A7). After cells were washed, they were run on a BD LSR II instrument and analyzed using FlowJo software, version 9.3 (Tree Star, Inc.).

Quantification of productive and abortive infectious virions. Cells were grown to confluence on glass coverslips and then infected with the viruses indicated in the figure legends diluted in BSS-BSA at an MOI of <0.01. After 1 h of adsorption, the inoculum was removed, and cells were overlaid with standard plaque assay overlay medium plus 0.9% agarose. After 15 h, overlays were removed, and cells were washed extensively with PBS and then fixed for 20 min in PBS–3.2% paraformaldehyde. Coverslips were then stained and imaged according to the immunofluorescence procedure outlined above. The ratio of nonpropagating to propagation-competent infectious virions was determined by eye using an epifluorescence microscope and was calculated for each coverslip examined by counting 100 to 500 infection events. Lone IAV protein-expressing cells or two adjacent IAV protein-expressing cells, surrounded by unstained cells, indicated infection with nonpropagating infectious virions. Infection with propagation-competent virions was indicated by foci of three or more adjacent IAV protein-expressing cells. IAV protein-expressing cells that were technically surrounded by unstained cells yet were situated in close proximity to infected foci were not counted.

RESULTS

Low-multiplicity IAV infection results in cells expressing an incomplete set of viral proteins. If packaging a complete set of vRNA segments is highly efficient, then the vast majority of infected cells should express all IAV gene products. A previous study, however, suggested that this might not always be the case (8). To test this prediction, we infected MDCK cells with A/Puerto Rico/8/34 (H1N1) (PR8) at different MOIs (based on $TCID_{50}$ titers) and simultaneously assessed the expression of HA, NA, and NS1 by immunofluorescence (IF) at 7 h postinfection (p.i.).

As expected, virtually all cells infected at a high MOI (≥1) expressed each of the three IAV proteins examined (Fig. 1A). When, however, we infected cells at an MOI of 0.04 to ensure single virion infection, substantial numbers of cells expressed one or two but not all three viral proteins at detectable levels. Thus, in the absence of viral complementation, a sizable fraction of IAV virions express an incomplete set of viral proteins.

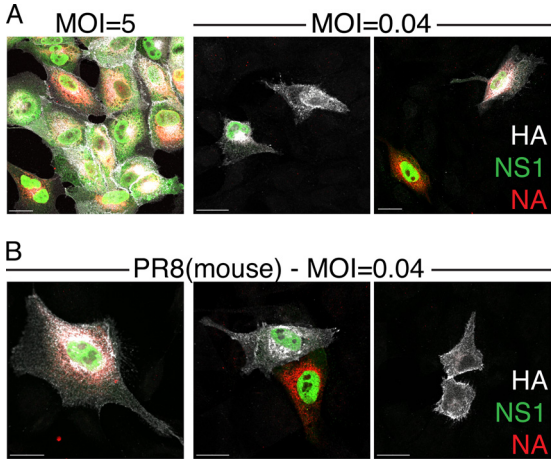

FIG 1 Low-multiplicity IAV infection results in cells expressing an incomplete set of viral proteins. (A) MDCK cells were infected with PR8 at MOIs of 5 and 0.04. At 7 h p.i. cells were fixed, permeabilized, and assessed for HA (human MAb 70-1F02), NA (rabbit pAb), and NS1 (mouse MAb 1A7) expression by IF. Images were acquired using a 40× objective and are representative of six independent experiments. (B) MDCK cells were infected with PR8 harvested from infected mouse lung at an MOI of 0.04 based on $TCID_{50}$ titer. At 7 h p.i., cells were assessed for HA, NA, and NS1 expression by IF as described for panel A. Images were acquired using a 63× objective and are representative of four independent experiments. Scale bar, 20 μm.

Incomplete infectious virions are produced *in vivo*. The PR8 stocks used in the experiment shown in Fig. 1A were grown in embryonated chicken eggs, clearly different from the respiratory epithelial cells most relevant to mammalian influenza virus transmission. IAV propagation in eggs can generate defective-interfering (DI) virions with vRNA segments exhibiting large internal deletions, typically in the polymerase genes (9–11). Do IAV virions propagated under more physiological conditions demonstrate a similar pattern of incomplete protein expression?

We infected C57BL/6 mice intranasally with 100 $TCID_{50}$s of PR8 and directly infected MDCK cells at a low MOI with virus present in 7-day lung homogenates. After 7 h, we assessed HA, NA, and NS1 expression levels as above (Fig. 1B). As with egg-grown virus, many lung-derived virions failed to express of the full set of viral proteins at detectable levels, demonstrating that the phenomenon extends to virions produced in the mammalian respiratory tract.

Incomplete viral protein expression is not observed during low-multiplicity VSV infection. Is the failure to express a complete set of viral proteins a general feature of RNA viruses? To address this question, we infected MDCK cells with vesicular stomatitis virus (VSV; negative-stranded, nonsegmented RNA genome). Five hours after infecting cells at an MOI of less than 0.01, we assessed expression of the viral G and N proteins by IF (Fig. 2). In contrast with IAV, VSV-infected cells lacking detectable expression of G or N were not observed among thousands of infected cells examined. Thus, in contrast to IAV, a very large fraction of individual VSV virions express their gene products as a complete set.

The majority of single-virion-infected cells express an incomplete subset of viral proteins. We turned to flow cytometry to

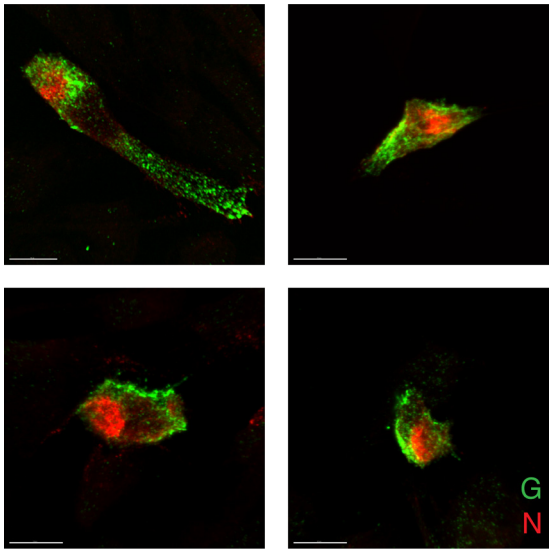

FIG 2 Incomplete viral protein expression is not observed during low-multiplicity VSV infection. MDCK cells were infected with VSV at an MOI of <0.01. At 5 h p.i. cells were fixed, permeabilized, and assessed for expression of the viral G (mouse MAb 8G5-F11) and N (rabbit pAb) proteins by IF. Images were acquired using a 63× objective and are representative of two independent experiments. Scale bar, 10 μm.

more precisely quantitate the frequency of cells coordinately expressing HA, NA, NP, and NS1 (Fig. 3). We infected MDCK cells with PR8 at MOIs of 0.03 to 0.05 for 16 h to maximize viral protein expression levels prior to staining (note that with the exception of PB1-F2 [12], IAV proteins exhibit high metabolic stability). To prevent second-round replication, at 2 h p.i. we added an anti-HA MAb with high neutralizing potency that binds noncompetitively with the anti-HA MAb used subsequently to measure HA expression.

In assessing HA and NA expression, infected cells were easily separable into HA- and NA-positive (HA^+ NA^+), HA-positive and NA-negative (HA^+ NA^-), HA^- NA^+, and HA^- NA^- populations (Fig. 3A). These populations were then further assessed for NS1 and NP expression (Fig. 3B). Cells were considered infected if they stained positive for at least one viral protein. Importantly, the variation in the levels of individual proteins was relatively narrow when the protein was expressed, facilitating discrimination between expressing and nonexpressing cells. Fewer than half (46.9%) of infected cells expressed 4/4 proteins examined (Fig. 3C). Notably, increasing the MOI to 1 or more resulted in a dramatic increase in the frequency of infected cells expressing a complete set of viral proteins, demonstrating that complementation restores complete viral protein expression, as expected (Fig. 3D).

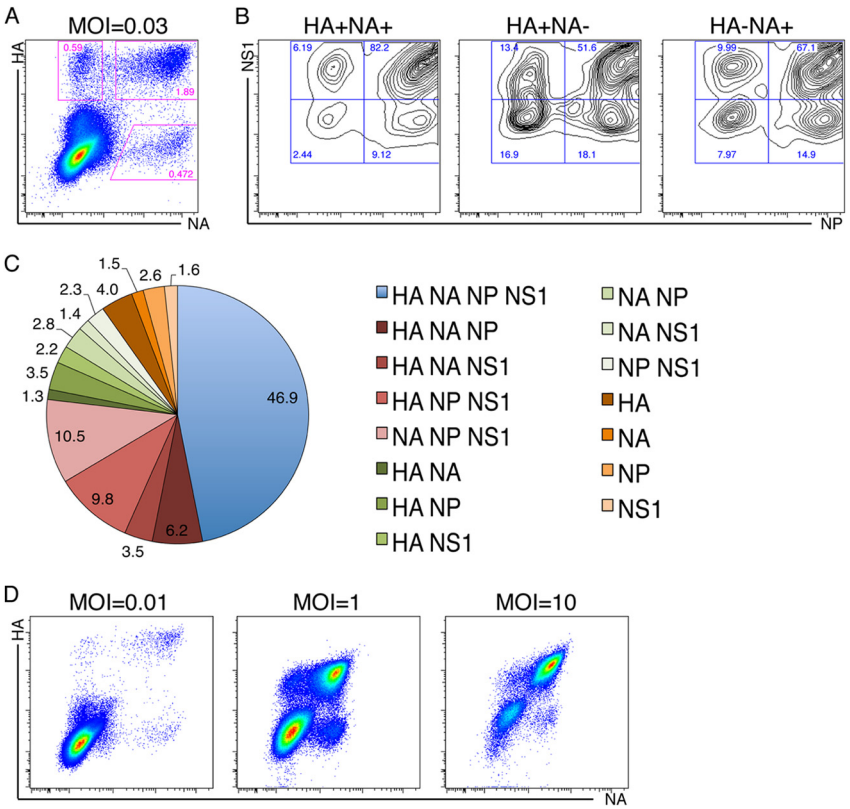

FIG 3 The majority of infectious IAV virions express an incomplete set of viral proteins. MDCK cells were infected in triplicate with PR8 at an MOI of 0.03. Two hours later, neutralizing anti-HA MAb (H17-L2; 10 μg/ml) was added to cultures. At 16 h p.i., cells were harvested, fixed/permeabilized, stained against HA (mouse MAb H36-26), NA (mouse MAb NA2-1C1), NP (mouse MAb HB-65), and NS1 (mouse MAb 1A7), and then run on an LSR II flow cytometer. (A) Representative dot plots from mock- and PR8-infected MDCK cells assessed for HA and NA expression. (B) Representative NS1 and NP expression histograms of the populations defined in panel A based on HA and NA expression. (C) Infected cells (based on expression of one or more of the viral proteins examined) were classified based HA, NA, NP, and NS1 expression patterns, and the percentage of the total infected cell pool that each of these populations made up was then calculated. Values represent the means of three infection replicates and are representative of five independent experiments. (D) MDCK cells were infected with PR8 at MOIs of 0.01, 1, and 10 and then analyzed as described for panel A.

TABLE 1 Expression of different IAV gene products is linked

Protein expression status	Correlating protein expression[a]							
	HA		NP		NA		NS1	
	Frequency (%)	% Reduction	Frequency (%)	% Reduction	Frequency (%)	% Reduction	Frequency (%)	% Reduction
HA^+			85.9 ± 0.2		74.9 ± 0.0		80.7 ± 0.7	
HA^-			80.2 ± 1.5	6.6 ± 1.9	71.2 ± 0.8	4.9 ± 1.1	69.8 ± 0.6	13.6 ± 0.8
NP^+	78.4 ± 0.5				78.5 ± 0.3		82.2 ± 0.7	
NP^-	70.8 ± 1.6	9.8 ± 2.5			49.7 ± 1.8	36.7 ± 2.5	56.6 ± 0.8	31.2 ± 1.2
NA^+	89.6 ± 0.4		89.7 ± 0.4				84.2 ± 0.7	
NA^-	74.7 ± 0.5	16.6 ± 0.8	70.2 ± 1.2	21.8 ± 1.5			61.2 ± 0.5	27.3 ± 0.8
$NS1^+$	79.7 ± 0.1		88.9 ± 0.2		79.7 ± 0.1			
$NS1^-$	68.4 ± 0.9	14.2 ± 1.1	69.2 ± 1.2	22.1 ± 1.4	53.7 ± 1.2	32.6 ± 1.6		

[a] MDCK cells were infected in triplicate with PR8 at a MOI of 0.03. Two hours later, neutralizing anti-HA MAb (H17-L2; 10 μg/ml) was added. At 16 h p.i. cells were harvested, fixed/permeabilized, simultaneously stained for HA (mouse MAb H36-26), NA (mouse MAb NA2-1C1), NP (mouse MAb HB-65), and NS1 (mouse MAb 1A7), and run on an LSR II flow cytometer. Looking within the total population of infected cells, we assessed the association between the expression status of each of the examined viral proteins and the expression frequency of the others. Values represent the mean of three replicates plus or minus the standard deviation and are representative of three independent experiments.

We suspect that the failure of some cells to achieve high levels of expression of HA or NA following infection at an MOI of 10 may result from defective-interfering (DI) particle activity, particularly since each cell is exposed to ~100 particles.

These data clearly demonstrate that a large majority of cells infected with single virions fail to express products from one or more gene segments.

Selective expression reveals gene segment linkages. The different genome segments of IAV do not operate independently. NP and NS1 are both required for maximal viral RNA replication and protein synthesis (13–15). Furthermore, a substantial body of evidence supports nonstochastic packaging of the eight vRNA segments; i.e., segments are packaged as positively interacting units (16–19). By comparing the expression frequencies of a given viral gene product between infected cells that do or do not express a different viral gene product, we can assess the interdependence between the two during the viral life cycle.

We therefore examined flow data to correlate the expression of each of the gene products measured with each other. For each viral protein, we divided infected cells into positive and negative populations and then compared the expression frequencies of the other viral proteins between the two (Table 1).

This analysis reveals that the failure to express a given viral protein is associated with variable reductions in the expression frequencies of other viral proteins. The failure to express HA was associated with a minor reduction in the expression frequencies of NP, NA, and NS1 (6.6%, 4.9%, and 13.6%, respectively). In concordance, failing to express NP, NA, or NS1 was associated with modest reductions in the expression frequencies of HA (9.8%, 16.6%, and 14.2%, respectively). In contrast, failure to express NP was associated with dramatic reductions in the expression frequencies of NA and NS1 (36.7% and 31.2%, respectively). The absence of NS1 expression was also associated with major reductions in the frequency of NA expression (32.6%) as well as NP expression (22.1%).

Together, these results demonstrate that the expression probabilities of different IAV gene products are linked, consistent with specific functional associations between segment pairs. Though we observed the largest effects with NP or NS1 expression, both of which are required for maximal viral gene expression (13–15), the relatively minor association between their expression and HA expression suggests that we are not simply observing global effects of the NP and NS genes on viral RNA replication and protein synthesis.

An incomplete protein expression pattern is observed in multiple target cell types. The absence of expression of individual gene products could be intrinsic to virions or reflect the efficiency of proper segment trafficking following viral penetration. In the latter case, we would predict cell line-dependent differences in gene product expression.

We therefore used flow cytometry as described above to measure PR8 HA and NA expression levels during low-multiplicity infections of MDCK, Vero, and A549 cells (Fig. 4). Vero cells have a deficient type I interferon (IFN) system (20, 21), also providing a test for the participation of IFN-dependent antiviral responses in restricting viral protein expression.

Each cell type exhibited clear populations of HA^+ NA^- and HA^- NA^+ cells. The relative percentages of HA^+ NA^+, HA^+ NA^-, and HA^- NA^+ cells were indistinguishable between MDCK and Vero cells, demonstrating that type I IFN induction is not required to limit expression of individual gene products. A549 cells exhibited a small but statistically significant reduction in the percentage of infected cells that were HA^+ NA^+, consistent with a minor role for the target cell in influencing the gene expression potential of IAV virions in some cases.

The majority of infectious virions are unable to produce infectious progeny. The existence of a substantial fraction of virions unable to express one or more viral proteins predicts that many virions are capable of only abortive infection. To quantitate virion infectivity, we infected MDCK monolayers on coverslips with PR8 at an MOI of <0.01 and overlaid cells with 0.9% agarose to limit free-virus spread. We incubated cells for 15 h, a period that allowed both virus propagation and visualization of viral protein expression in cells infected in the first round of replication. Staining cells for HA, NP, M1, and M2 allowed us to quantify the number of cells infected in the initial round of viral replication and to determine the fraction of infection events resulting in release of infectious progeny.

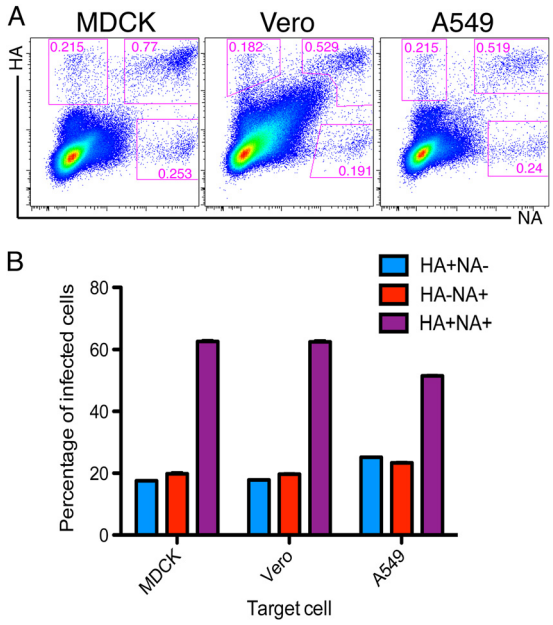

FIG 4 An incomplete viral protein expression pattern is seen in multiple target cells. MDCK, Vero, and A549 cells were infected in triplicate with PR8 at an MOI of 0.03. Two hours later, neutralizing anti-HA MAb (H17-L2; 10 μg/ml) was added to cultures. At 16 h p.i. cells were harvested, fixed/permeabilized, stained against HA (mouse MAb H36-26) and NA (mouse MAb NA2-1C1), and then run on an LSR II flow cytometer. (A) Infected cells (based on expression of one or more of the viral proteins examined) were classified based on HA and NA expression patterns. (B) The percentage of the total infected cell pool that each of these populations made up was then calculated. Data represent the means ± standard errors of the means of three infection replicates and are representative of two independent experiments.

IF revealed well-defined infectious foci (Fig. 5A), clearly indicative of multiple rounds of viral replication. Much more commonly observed, however, were lone infected cells and pairings of adjacent infected cells (likely to represent mitotic daughter cells). Quantitating lone infected cells/pairs versus foci, we determined that only 12.5% of infectious events led to viral propagation (Fig. 5B). Similarly, only 11% of infectious foci in Vero cells demonstrated viral propagation, ruling out a role for type I IFN in limiting viral gene expression.

These findings indicate that nearly 90% of infectious IAV virions are incapable of producing infectious progeny, independent of target cell type or the presence of an intact type I IFN system.

The packaging cell does not influence propagation efficiency. What role does the packaging cell environment play in determining the fraction of virions capable of supporting productive infection? We expanded PR8 in eggs, MDCK cells, mice, and guinea pigs and compared the percentages of propagation-competent infectious virus. Remarkably, the source of virus had no significant effect on the fraction of propagation competence (Fig. 5C). This indicates that viral infectious potential is a relatively stable feature that is not typically affected by the producing cell.

Virions from multiple genotypes express an incomplete set of proteins. Given the extensive passage history of PR8 in ferrets, mice, and eggs, PR8 might not be representative of naturally circulating strains of H1N1, let alone other IAV subtypes. To generalize our findings, we assessed patterns of viral protein expression during low-multiplicity infection with a low-passage-number hu-

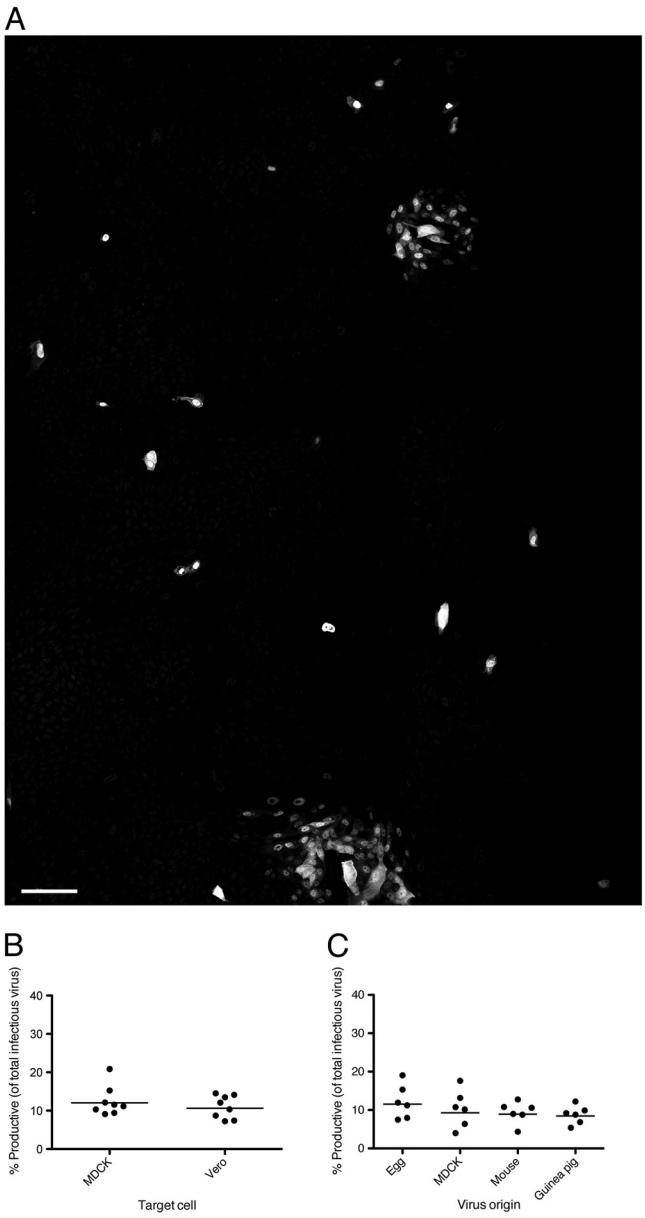

FIG 5 The majority of infectious IAV virions are incapable of producing infectious progeny. Confluent monolayers were grown on coverslips, infected with the indicated viruses at an MOI of <0.01, and then overlaid with 0.9% agarose. After 15 h, cells were fixed, permeabilized, and then stained against the indicated viral proteins. (A) MDCK cells were infected with PR8 and then stained against HA (mouse MAb H36-26), NP (rabbit pAb), and M1/M2 (goat cross-reactive pAb) (all false-colored white). The confocal image was generated with a 40× objective by tiling *z*-stacks and is representative of 12 independent experiments. Scale bar, 100 μm. (B) MDCK and Vero cells were infected with PR8 and then stained against HA (mouse MAb H36-26) and NP (rabbit pAb). Between 100 and 400 infection events on each coverslip were counted by eye as either abortive (1 to 2 adjacent, lone infected cells) or productive (3 or more adjacent infected cells), and the percentage of infection events that were productive was calculated. Each data point represents the value generated from a single coverslip, and the bars represent the means. (C) MDCK cells were infected with stocks of PR8 grown in eggs and MDCK cells, lung homogenates from PR8-infected mice, and nasal wash from PR8-infected guinea pigs and assessed as described for panel B. Data are representative of two independent experiments.

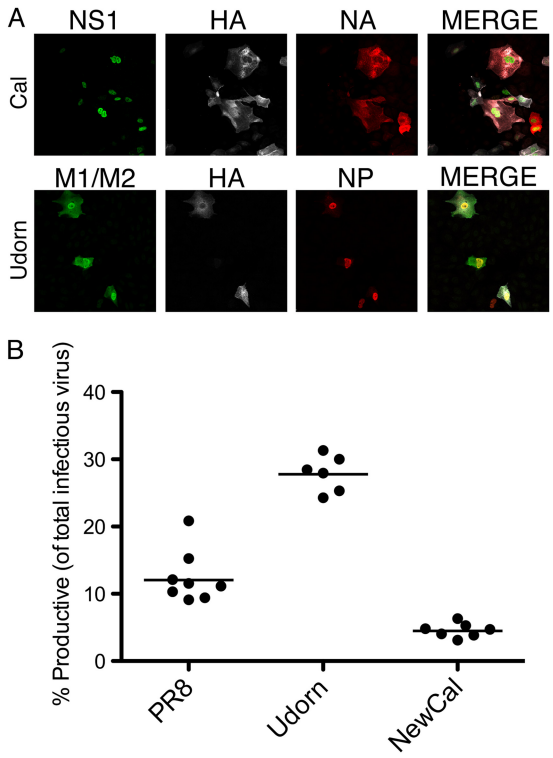

FIG 6 The efficiency of propagation-competent virus production differs widely between different viral genotypes. (A) A/Udorn/307/72 (Udorn) and A/California/07/09 (Cal) were grown in embryonated hen eggs and used to infect MDCK cells at MOIs of 0.01 to 0.03. At 7 (Udorn) or 16 (Cal) h p.i., cells were fixed, permeabilized, and assessed for HA (mouse MAb H14-A2), NP (rabbit pAb), and M1/M2 (goat cross-reactive pAb) (for Udorn) or HA (human MAb 70-1F02), NA (rabbit pAb), and NS1 (mouse MAb 1A7) (for Cal) expression by IF. Images were acquired using a 40× objective and are representative of three independent experiments. (B) Confluent monolayers of MDCK cells were grown on coverslips, infected with the indicated viruses at an MOI of <0.01, and then overlaid with 0.9% agarose. Fifteen hours later, cells were fixed, permeabilized, and then stained against HA (human MAb 70-1F02) and NP (rabbit pAb). Between 100 and 400 infection events on each coverslip were counted by eye as either abortive (1 to 2 adjacent, lone infected cells) or productive (3 or more adjacent infected cells), and the percentage of infection events that were productive was calculated. Each data point represents the value generated from a single coverslip, and the bars represent the means. Data are representative of four independent experiments, using multiple stocks of each virus grown in eggs and MDCK cells.

man isolate from the 2009 H1N1 pandemic, A/California/07/09 (Cal), as well as with the H3N2 strain A/Udorn/307/72 (Udorn). At 7 (Udorn) or 16 (Cal) h after infecting MDCK cells at MOIs of 0.01 to 0.03, we assessed fixed and permeabilized cells for viral protein expression by immunofluorescence (Fig. 6A). As with PR8, many cells infected with either Cal or Udorn exhibited incomplete viral protein expression patterns, demonstrating that the production of incomplete infectious virus is a general feature of IAV biology.

The efficiency of propagation-competent virus production differs widely between different viral genotypes. Expanding the above results, we infected MDCK coverslip monolayers with PR8, Udorn, or A/New Caledonia/20/99 (NewCal) at an MOI of <0.01 and then overlaid cells with 0.9% agarose and quantitated abortive versus productive infectious events as described above (Fig. 6B). When we compared the different viruses, we observed a nearly 10-fold range in the proportion of infectious virus that was propagation competent ($P < 0.0001$, by analysis of variance [ANOVA]). Roughly 30% of infectious Udorn virions were capable of yielding infectious progeny, compared with less than 5% for NewCal. These results were obtained reproducibly, using multiple virus preps grown in eggs and MDCK cells. Thus, different IAV genotypes differ widely in their abilities to produce propagation-competent virus.

DISCUSSION

As is the case for many viruses, the vast majority of IAV particles register as noninfectious by traditional, limiting-dilution assays. Often, this population of "noninfectious" virus is dismissed as a combination of dead-end by-products of faulty assembly and DI particles. While a few studies have suggested that the universe of IAV particles is more diverse, incorporating additional incomplete or abortive infectious forms of the virus, little attention has been paid to the biological potential of the noninfectious particle population (8, 22).

Here, by analyzing the outcome of single-hit IAV infection, we describe a population of infectious IAV virions that fail to express detectable levels of one of more viral proteins and, consequently, are unable to support multiround viral replication. We thus term this population of virions "semi-infectious" and show that it greatly outnumbers the propagation-competent population of virions. We further demonstrate that the propagation-competent fraction of virions varies widely between different strains of IAV. These results, which are the first to our knowledge to quantitatively examine the protein expression potential of individual IAV virions, demonstrate that semi-infectious virions are the primary product of IAV infection.

We assessed the infectious potential of individual virions by two means: protein expression and ability to propagate. If these two observations are related, they should be mathematically compatible. We were able to determine the expression frequencies of proteins from four of the eight vRNA segments. By averaging expression frequency from a representative experiment (frequency of HA expression [f_{HA}], 75.3%; f_{NA}, 77.3%; f_{NP}, 81.2%; f_{NS1}, 78.4%; $f_{average}$, 78.1%) and extending it to the four unexamined gene segments, we can estimate the frequency of virions expressing products of all eight vRNA segments ($f_{complete}$) as follows: $f_{complete} = (f_{HA})(f_{NA})(f_{NP})(f_{NS1})(f_{average}^4)$. For the representative experiment, $f_{complete} = (0.753)(0.773)(0.812)(0.784)(0.781^4) = 0.138$, or 13.8%.

This result agrees well with our finding that 12.5% of infectious PR8 virions are propagation competent in MDCK cells. Calculating in reverse and assuming that the 12.5% of infectious PR8 virions that are propagation competent represent those virions capable of expressing all gene products, solving for x in the equation $x^8 = 0.125$, where x represents the average expression frequency from the different genome segments, yields $x = 0.771$, or 77.1%. Again, this agrees well with our experimentally obtained average of 78.1%. Thus, these two measures of viral dysfunction corroborate each other and suggest that incomplete viral protein expression is the main cause underlying the inability of most IAV virions to propagate.

IAV typically initiates rapid, high-yield expression of its structural gene products upon fusing with a host cell. In this study, we detected viral protein expression by immunostaining with a collection of strain-specific and cross-reactive MAbs and sera, chosen

because they produced clear separation between positive and negative populations for the specific viruses used. Since every experimental method has a limit of detection, we cannot absolutely rule out miniscule levels of expression; however, the data clearly show that most IAV virions fail to express one or more viral gene products to anything approaching the levels associated with normal infection.

Virions that express an incomplete set of viral proteins can be explained by at least four, non-mutually exclusive mechanisms, as follows.

First, segments encoding missing protein(s) could result from internal deletions that prevent expression of functional gene products, thus resembling classic DI RNAs (9, 23). Our finding that the expression frequencies of the four gene products tested were roughly comparable (75.3% to 81.2%) and independent of segment length suggests that DI RNA formation is not a major contributor as the frequency of internal deletions for a given vRNA segment is reported to be proportional to the length of that segment (24). However, much remains unknown about DI dynamics during IAV infection, and more work is necessary to understand the role of DI RNAs in semi-infectious virus production.

Second, gene product expression failures could result from non-sense or other lethal mutations. The reported IAV mutation frequency of $\sim 10^{-5}$ to 10^{-6} per nucleotide (25) suggests that segments bearing non-sense mutations or lethal amino acid substitutions cannot explain the observed ~20 to 25% of infectious particles that fail to express a given gene product.

Third, incoming RNPs may not be successfully transcribed. Although incoming RNPs seem to traffic to nuclei (the site of mRNA transcription) at high efficiency, the success rates for trafficking and transcribing individual RNPs are unknown (26, 27). The differences observed in the percentage of infected cells that expressed both HA and NA in A549 versus MDCK and Vero cells suggests that the success rate of these various postentry events may differ between different host cells in some cases.

Fourth, and simplest, virions may fail to package one or more vRNA segments. Though a recent study provides solid evidence for efficient PR8 genome packaging, we cannot absolutely rule out a role for packaging failure (6).

Numerous studies highlight the essential role of NP in viral RNA replication, so it was surprising that we detected nearly normal levels of viral protein synthesis in cells lacking detectable NP expression (15, 28, 29). While the expression of the other viral gene products was diminished to various degrees in the absence of NP, these results clearly demonstrate that detectable *de novo* synthesis of NP is not an absolute requirement for robust viral protein expression. It may be that the incoming RNP-associated NP (~1,000 copies/virion) is sufficient to drive primary mRNA transcription and that primary transcription is sufficient for robust viral protein synthesis. Alternatively, infected cells that fail to produce detectable levels of NP may still synthesize sufficient NP to support viral protein synthesis.

Our finding that the fraction of propagation-competent virions varies nearly 10-fold between different strains indicates that this trait is subject to evolutionary selection and implies that generating replication-incompetent virions may be advantageous in some circumstances. At low MOIs, as would be expected early during natural transmission events, semi-infectious virion production would be detrimental on the basis of nonpropagation though this effect could be mitigated by aggregation-mediated complementation (30). At high MOIs, however, the inability of a virion to express a full complement of gene products should have a minimal negative effect due to complementation. Here, the production of complementation-dependent virions could be advantageous in imposing a step that mandates the mixing of segments from different input viruses, thereby increasing the frequency of genetic reassortment.

At a minimum, our results reveal that semi-infectious virions can dramatically outnumber the propagation-competent virus detected by $TCID_{50}$ or plaque assay. The extent to which these assays underrepresent the true infectious potential of a virus population is dependent on the relative proportions of infectious and semi-infectious virus and thus will differ from strain to strain. This must be taken into account when trying to interpret the behavior of different IAV strains under conditions of low-multiplicity infection, such as those encountered during animal-animal transmission.

Together, these findings suggest that IAV effectively exists less as a population of intact virus than as a swarm of complementation-dependent, semi-infectious virions. Understanding the role that semi-infectious virion production plays during IAV infection will be key in further expanding our understanding of IAV pathogenicity and transmissibility.

ACKNOWLEDGMENT

This work was generously supported by the Division of Intramural Research, NIAID.

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AUTHOR'S CORRECTION

Most Influenza A Virions Fail To Express at Least One Essential Viral Protein

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Volume 87, no. 6, p. 3155–3162, 2013. Page 3157, Fig. 3D: The *x* axis should be labeled "NP."

Page 3157, legend to Fig. 3, line 8: "and then analyzed as described for panel A" should read "and then assessed for HA and NP expression."

These changes in no way affect the interpretation of the data or the conclusions.

doi:10.1128/JVI.01308-13